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# THE DUAL NATURE OF RESISTANCE MECHANISMS AS REVEALED BY STUDIES OF ANTHRAX SEPTICEMIA

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## ANIMAL RESEARCH

In conducting the research reported herein, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society of Medical Research.

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## ABSTRACT

From studies of septicemic anthrax, resistance is described in relation to toxin and to growth of bacilli. This description is based on the observations that the terminal concentrations of bacilli in the blood are influenced primarily by the susceptibility of the host to toxin, whereas the death response time of the host is dependent on both resistance to bacilli and toxin susceptibility. Resistance to the establishment and growth of infecting organisms and susceptibility to the toxin produced by growth of the bacilli are separate aspects of pathogenesis. A complete description of pathogenesis must treat accordingly both these phenomena as individual entities.

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## I. INTRODUCTION

Although it is generally agreed that a septicemia, i.e., a progressing bacteremia, is observed with anthrax, quantitative data are scarce. Keppie et al[1] and Klein et al[2] present data for guinea pigs; Trnka et al[3] give data for sheep. Observations on four chimpanzees and four rhesus monkeys, respectively, are given by Albrink and Goodlow[4] and Middleton and Stanton.[5] In contrast, Stockman[6] and Bloom et al[7] suggest that a bacteremia remains slight in immunized animals. Thus, complete detailed data on the occurrence of bacteremia and/or septicemia in anthrax are currently available only for the disease in guinea pigs. There has been a logical reluctance to assume that the observations on guinea pigs apply to other species, and particularly species that seem to react differently to infection by B. anthracis than the guinea pig does.

Bennet and Beeson[8] showed that the septicemic course of a disease is altered by several factors, including the parasite virulence and the host's ability to remove bacteria. They point out the need for accurate comparison of results in normal animals with those in animals subjected to various types of treatment or manipulation.

In line with this thought Klein et al[2] showed that guinea pigs innunized with alum-precipitated antigen differed from control guinea pigs in two ways. One was an increased resistance that allowed immunized animals to withstand 1600 times more challenge organisms than controls. The other was that the terminal concentration of bacilli in the blood of immunized animals succumbing to challenge was only about one sixth that of controls and independent of the size of the challenge dose. It was also shown that resistance, as measured by time-to-death following challenge, was reduced by egg-yolk treatment of the challenge spores. Yet the septicemic course of anthrax, including the terminal concentration of bacilli, was unaltered by this treatment of the spores.

Observations are recorded on the rate of septicemic development and the terminal level of organisms in the blood of the naturally resistant rat and the naturally susceptible mouse. Egg-yolk enhancement of virulence was studied in each host while age and strain were varied in the rat. These studies show that resistance to anthrax must be described both in relation to the ability to establish growth in a host, to the susceptibility of the host to toxin produced during growth of the bacilli.

## II. EXPERIMENTAL PROCEDURE

### A. MATERIALS AND METHODS

#### 1. Infecting Organism

The Vollum strain (Vib) of B. anthracis was used and grown in N-Z-Amine-Type A (Sheffield Chemical Co., Norwich, New York) medium at 34°C for 24 hours.

#### 2. Animals

Black rats obtained from the National Institutes of Health Animal Farm, where they were developed from Long-Evans stock and Fischer 344 strain of albino rat,[9] were used in this investigation. Swiss mice were obtained from the Fort Detrick Animal Farm.

#### 3. Virulence Enhancement

This was achieved by treating anthrax spores with egg yolk.[10]

#### 4. Blood Assay of Septicemia

Concentration of organisms per milliliter of whole blood was obtained by microscope counts of the stained organisms from a measured amount of blood smeared on a one-square-cm area of a slide.[1]

#### 5. Viable Counts

Surface plate counts were made from serial dilutions in gel-phosphate buffer on tryptose agar fortified by glucose (0.9 per cent) and agar (0.5 per cent). Colonies were counted after 24 hours' incubation at 34°C.

#### 6. In Vitro Toxin

This was prepared as described by Thorne et al.[11]

#### 7. Antiserum

Hyperimmune antiserum was prepared in a horse by injecting washed spores of the Sterne strain. This is the same antiserum described by Thorne et al[11] and is designated as DH-1-4A.

#### 8. In Vivo Toxin

This was obtained from the lymph and terminal blood of monkeys challenged with anthrax spores of the Vollum strain. Processing of the lymph and blood was the same as described by Smith et al.[18]

9. Challenge

All animals were challenged by intraperitoneal injection of organisms treated as described.

## B. DESCRIPTION OF STUDIES

The results reported in this article came from three separate areas of work. One was designed to study the effect of virulence enhancement on septicemia in two host species. In a second experiment, differences in septicemia between different strains and ages of rats were studied. The third area provided information on the interaction between egg-yolk treatment and host age on the dose-response relationship of the rat.

In the first study, trials were repeated until techniques were established and observations on an adequate number of animals were built up for each treatment condition. In each trial, groups of animals were randomly assigned to treatments. The number of animals assigned to each treatment condition at the finish of the study are:

| Animal | No. of Animals | Treatment Condition of Inoculant |
|---|---|---|
| Rat | 30 | Spores only - high dose |
| | 30 | Spores only - low dose |
| | 8 | Spores mixed with egg-yolk high dose |
| | 8 | Spores mixed with egg-yolk low dose |
| Mouse | 12 | Spores only |
| | 12 | Spores mixed with egg yolk |

Starting at ten hours prior to expected death time of the rat and six hours prior to expected death time of the mouse, blood was withdrawn from the tail of each rat every three hours and from each mouse every two hours until death occurred. The time-to-death was recorded, and the sampling time prior to death was calculated.

The study to determine how the variables of the septicemia were influenced by age and strain was replicated. Ten rats of each strain, NIH Black and Fischer 344, and each age, 23, 44, 65, and 86 days, were challenged. Since the data from 65- and 86-day-old rats were not significantly different in any respect, these two groups were pooled and are referred to throughout the remainder of this paper as 76-day-old rats. Blood samples were drawn and assayed as described above.

The third study was an extension of the work of Taylor *et al*[9] to include Fischer and NIH rats at 30, 60, and 90 days of age. Three replicates of this study were run and data were obtained on 261 animals divided among the treatments. Each animal was challenged with $10^8$ egg-yolk-treated or nontreated spores; observations on death were made every half hour over a 48-hour period, after which the animals were observed every six hours for eight days.

## C. ANALYSIS

For all these studies the number of organisms per milliliter of blood were plotted according to the time prior to death for each infected animal. Preliminary plots of these data indicate that the bacilli in the blood increased exponentially with time. This relationship could be expressed mathematically as:

$$V_t = V_d \, 10^{-bt} \qquad (1)$$

where $V_t$ is the number of bacilli per milliliter of blood at any time t in hours prior to death, $V_d$ is the number of bacilli per milliliter of blood at death, and b is the hourly rate of increase of organism in the host's blood.

Least-squares estimates, $V_d$ and b, of the unknown parameters in Equation (1) were made from the data.

## III. RESULTS

The concentration of bacilli in the blood of the NIH rats during the interval from 14 hours prior to death to 2 hours prior to death is shown in Figure 1. Spores were not present as measured by test for the presence of heat-resistant bacteria. Since there was a variable number of observations at each time period, the data were combined to give geometric mean values for time intervals of two hours. The constants $V_d$ and b, calculated from the data for each of the challenge methods, are shown in the same figure beside the set of data to which they refer. Neither terminal concentration nor doubling time (slope) differed significantly among the three challenges. Thus, the average terminal concentration of organisms was calculated to be $V_d = 1 \times 10^6$. The 95 per cent confidence limits around this estimate extend from $0.6 \times 10^6$ to $1.6 \times 10^6$ organisms. The average doubling time of the bacilli in the blood, calculated from the estimated slope, is 120 minutes with 95 per cent confidence limits from 102 to 139 minutes. Direct counts were comparable with plate counts.

In mice, when the challenge dose consisted of untreated spores, heat-resistant spores were found in the blood samples throughout the course of the disease. When the challenge dose consisted of egg-yolk-treated spores, heat-resistant spores were rarely observed in the blood, even early in the development of the disease. In order to distinguish heat-resistant spores from germinated spores and vegetative cells (the latter two forms are collectively referred to as bacilli in this paper) two counts were made on each blood sample. The first was a count of total organisms in untreated blood. The second was a count for heat-resistant spores made from a heat-shocked sample of blood. The difference between these two counts represents an estimate of the concentration of vegetative anthrax organisms in the blood. Thus, bacilli/ml = total organism/ml - heat-resistant spores/ml.

The concentration of bacilli in the blood of mice during the interval from 7 hours to 30 minutes prior to death is presented in Figure 2. These data have been combined as geometric means associated with a time interval of one hour. As with rats, there were no significant differences between terminal concentrations or doubling time of bacilli, regardless of treatment conditions. Estimates of the constants for each treatment condition are shown in Figure 2. The average for all three treatments of the concentrations of bacilli in the blood at death is $V_d = 10 \times 10^6$ with 95 per cent confidence interval from $4 \times 10^6$ to $25 \times 10^6$. The average doubling time of 45 minutes with a 95 per cent confidence limit of 37 to 59 minutes was significantly reduced relative to the situation with rats. As with rats, direct counts were comparable with plate counts.

It became apparent from these data and experience with guinea pigs that the bacterial generation time and terminal concentration are not altered by egg-yolk treatment of the challenge anthrax organism. It appears from our observation on mice that virulence enhancement following

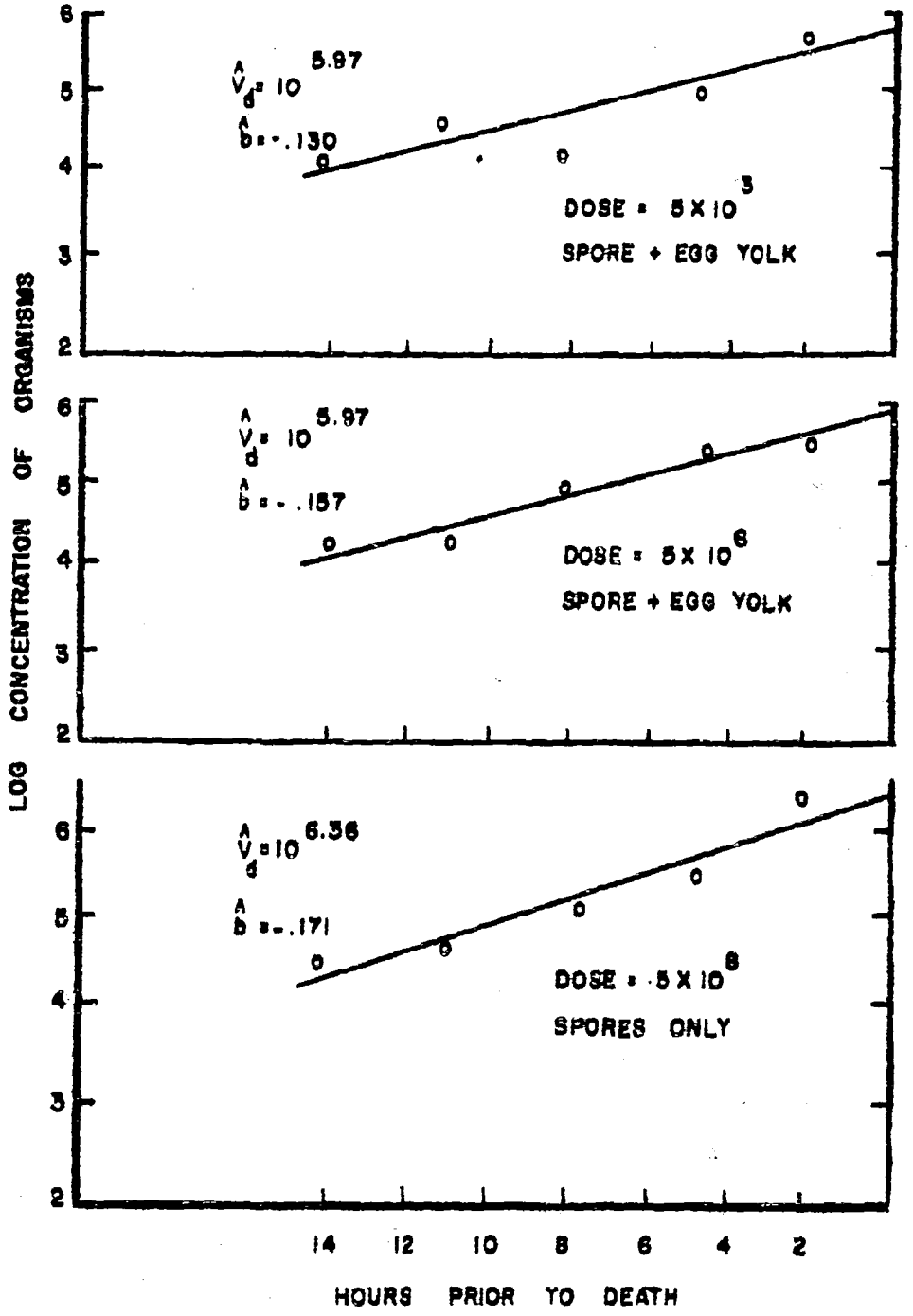


Figure 1. Effect of Dose and Treatment on Regression of Log Concentration of Organisms in Rat Blood on Time prior to Death.

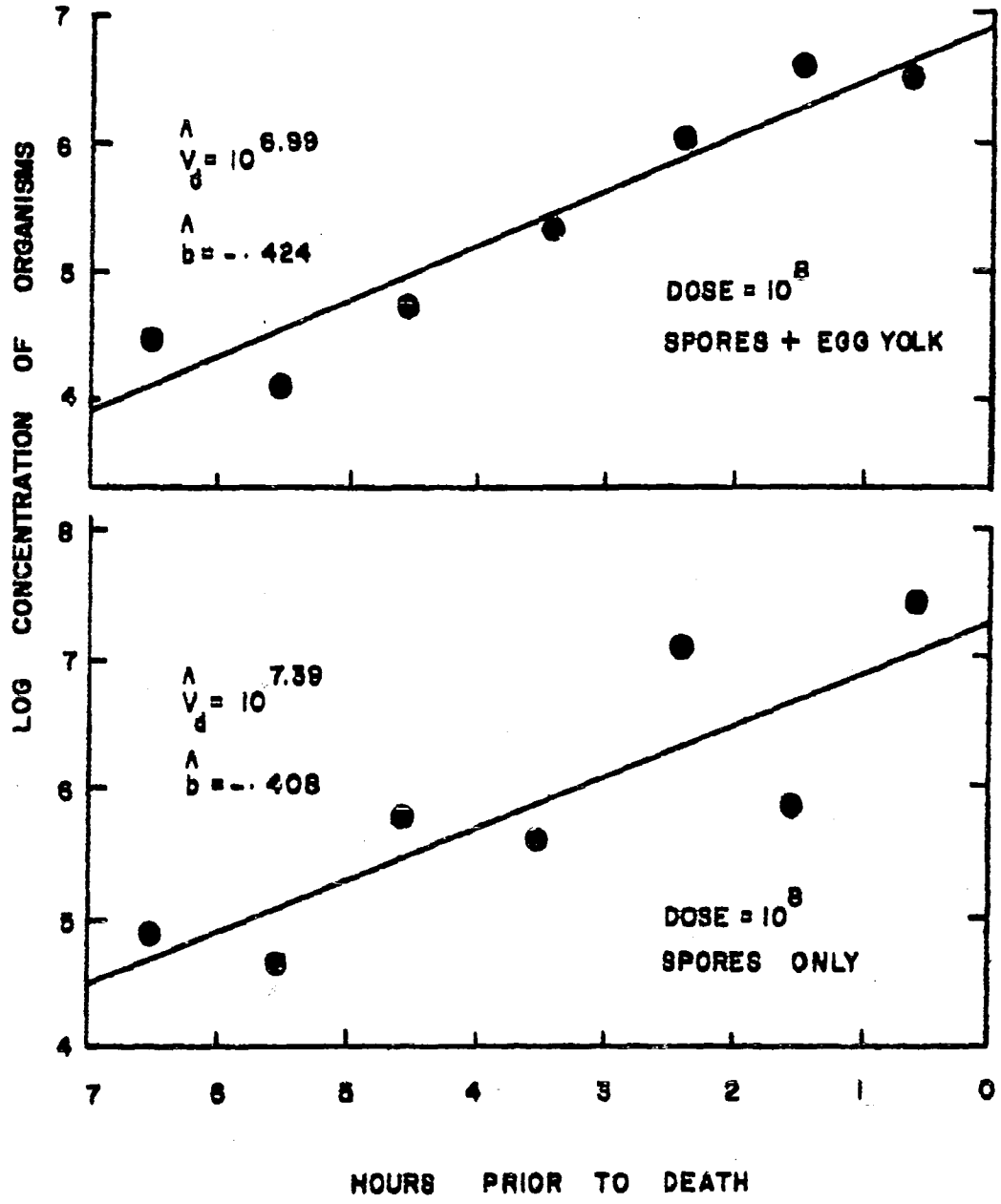


Figure 2. Effect of Treatment on Regression of Log Concentration of Organisms in Mouse Blood on Time Prior to Death.

egg-yolk treatment is, in part, the result of early germination of the spores. This observation supplements other observations of this phenomenon, namely: early and massive encapsulation which Mesrobeanu and Slavescu[13] have observed when spores plus egg yolk are inoculated into animals and which we have demonstrated in vitro when egg-yolk-treated spores and whole blood are incubated and examined microscopically; and a greatly increased retention of the inoculum in the peritoneum when egg yolk is present in the inoculum, a phenomenon that we have also observed through microscope studies of peritoneal fluid.

Any one or a combination of these three factors would enhance the invasive capacity of the organisms by increasing their ability to overcome the initial resistance of the host. Thus, it appears that egg yolk exerts its effect before bacterial multiplication and spread proceeds to the septicemic stage. By this time, the host defenses have been overcome and the resulting septicemia then progresses at a fixed bacterial generation rate to the same terminal concentration of bacilli per milliliter of blood, regardless of preliminary spore treatment. The fact that generation time and terminal concentration of bacilli in the blood appear to be constants, with a characteristic rate or level for each host species, may indicate that the degree of phagocytosis and multiplication of bacilli in tissues prior to septicemia are two important factors in the differences of resistance to anthrax of different host species.

In the second study, we observed differences in the septicemic stage of anthrax dependent upon the age of NIH rats and Fischer rats. Regression lines showing concentration of organisms in the blood of NIH rats are shown in Figure 3. Slopes of these lines plotted from plate counts are statistically significant at the 95 per cent level. Slopes for 23-day NIH rats and for all Fischer rats were not significantly different from zero, so they are not shown. Terminal concentrations of bacilli per milliliter of blood from all ages of both strains are shown on the vertical axis (as log concentration). All these estimates with their standard errors are presented in Table I.

From this study it is evident that there are marked differences in the terminal concentration of bacilli between these two strains of rats. The NIH black rat died with the higher terminal level and at a later time than the Fischer 344 rat. From observations of terminal concentration and doubling time, we learned that, at the same time that the 76-day-old Fischer rats were dying with a terminal concentration of $10^{3.8}$ organisms per milliliter, the blood of the 76-day-old NIH rats contained a significantly higher concentration of about $10^{5.5}$ organisms per milliliter.

If we assume that toxin production is continuous and proportional to the concentration of organisms, this observation indicates that the mature NIH rat is both less resistant to infection and less susceptible to toxin than the mature Fischer rat. The former must be true, because the concentration of organisms builds up faster and higher in the NIH than in the Fischer.

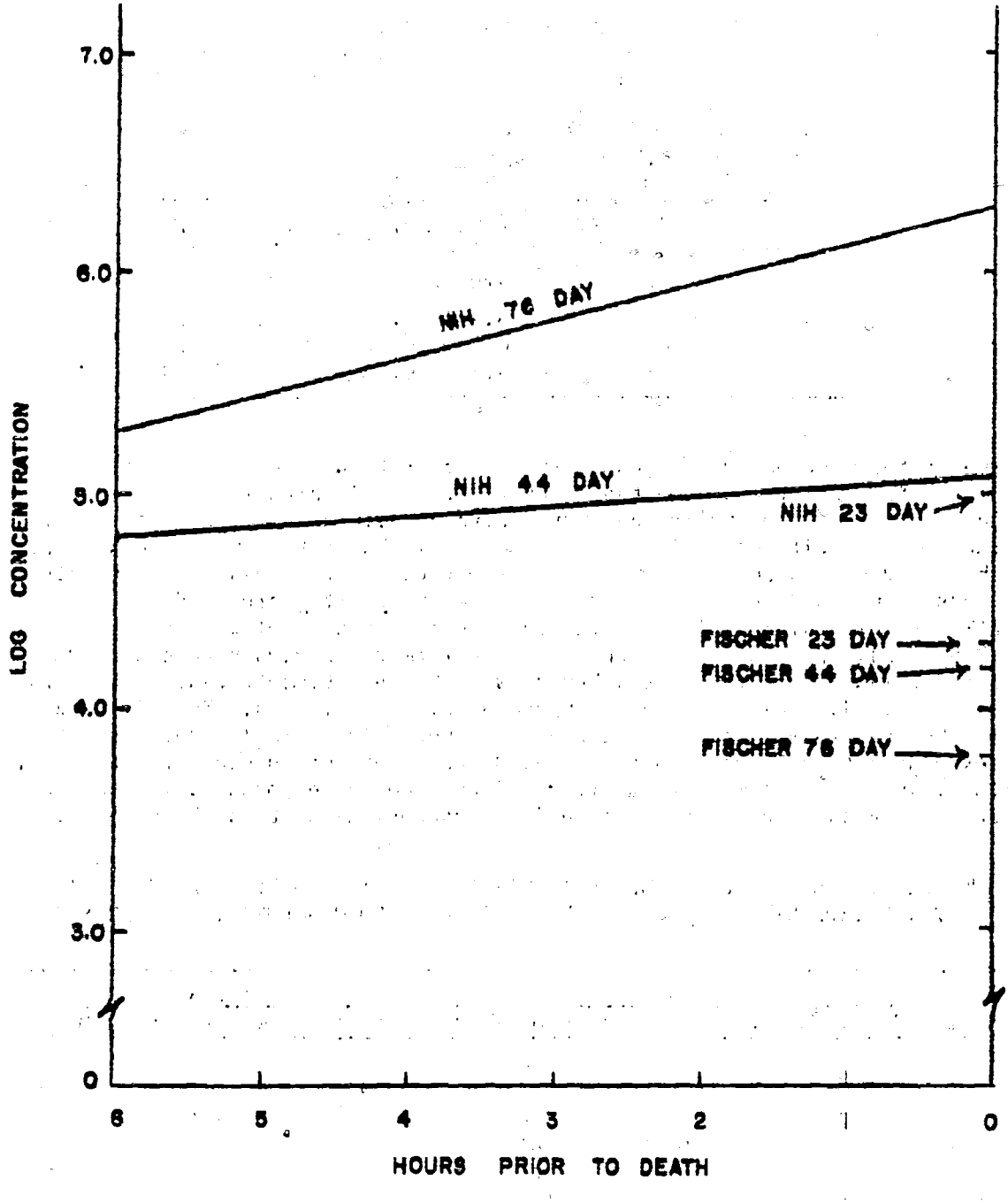


Figure 3. Concentration of B. anthracis Organisms per Milliliter of Blood by Age of Rat and Time Prior to Death.

TABLE I. ESTIMATES OF SLOPES OF SEPTICEMIA AND TERMINAL CONCENTRATION OF ORGANISMS IN THE BLOOD OF RATS BY TYPE AND AGE

| Type of Rat | Age of Rat, days | $V_d$ | S.E. | b | S.E. |
|---|---|---|---|---|---|
| NIH | 76 | 6.3 | 0.26 | 0.37 | 0.06 |
| | 44 | 5.1 | 0.14 | 0.17 | 0.01 |
| | 23 | 5.0 | 0.44 | 0.05 | 0.30 |
| Fischer | 76 | 3.8 | 0.11 | 0.01 | 0.02 |
| | 44 | 4.2 | 0.11 | 0.02 | 0.01 |
| | 23 | 4.3 | 0.14 | 0.05 | 0.03 |

The latter must be true, because the concentration of organisms in the blood of the NIH rat at the time the Fischer rat dies is high enough to have released an amount of toxin that would have killed the Fischer rat. Thus, the concentration of toxin that kills the Fischer rat is not enough to kill the NIH rat. These observations are supported by the results of experiments in which Fischer rats were killed with <u>in-vitro</u> produced toxin, but NIH rats were killed with a much higher concentration of the same material (Table II). In addition, terminal blood from 21 monkeys that died from anthrax was tested in 107 Fischer rats. The mean time-to-death of the 73 rats that died was 303 minutes, range 54 to 1260 minutes. In only three of the monkeys was the blood found to be nontoxic. Similar results with toxin have been shown by Stanley and Smith[14] between guinea pigs and mice. Thus, we interpret a host's pattern of septicemic response as indicative both of resistance to infection and to susceptibility to anthrax toxin.

TABLE II. MEAN TIME TO DEATH IN MINUTES OF RATS CHALLENGED WITH ANTHRAX TOXIN PRODUCED <u>IN VITRO</u> BY STRAIN OF RAT AND CONCENTRATION OF TOXIN

| | Fischer Rat | | | NIH Rat | | |
|---|---|---|---|---|---|---|
| Toxin | Total No. | No. Survived | MTD (Min) | Total No. | No. Survived | MTD (Min) |
| 10X | 0 | - | - | 22 | 12 | 2894 |
| 8X | 0 | - | - | 2 | 2 | S |
| 5X | 0 | - | - | 2 | 2 | S |
| 4X | 6 | 0 | 62 | 2 | 2 | S |
| 2X | 6 | 0 | 76 | 0 | - | - |
| Unconc | 8 | 0 | 105 | 4 | 4 | S |
| ½X | 8 | 1 | 446 | 4 | 4 | S |
| ¼X | 8 | 8 | S[a] | 2 | 2 | S |

In the third study we demonstrate that the effects on mean response time discussed in the previous two studies of egg-yolk treatment held true over the age range tested for both strains of rats. In Table III we show by strain and age of rat and egg-yolk treatment the harmonic mean time-to-death of the 261 animals tested. From this table it is seen that animals of both strains and all ages challenged with egg-yolk-treated spores died significantly sooner than animals challenged with nontreated spores. Since egg yolk is known to enhance the invasive capacity of the organism and block the resistance of the host, one would expect the effect of this treatment to be greater in a relatively more resistant host. This phenomenon is illustrated in Figure 4 as a greater distance between egg yolk and non-egg yolk lines for Fischer than for NIH rats. Thus the effect of egg-yolk treatment on the relatively more resistant (to infection) Fischer rat is greater than the effect of this treatment on the relatively less resistant NIH rat.

TABLE III. HARMONIC MEAN TIMES-TO-DEATH IN HOURS OF 261 RATS BY TYPE AND AGE OF RAT AND CHALLENGE TREATMENT

| Type of Rat | Challenge Treatment Dose $1 \times 10^9$ Spores | Age of Rat, days 30 MTD | 30 S.E. | 60 MTD | 60 S.E. | 90 MTD | 90 S.E. |
|---|---|---|---|---|---|---|---|
| Fischer | Spores + E.Y. | 5.7 | 0.15 | 8.3 | 0.15 | 9.1 | 0.15 |
| | Spores | 17.0 | 0.15 | 25.0 | 0.15 | 25.0 | 0.15 |
| NIH | Spores + E.Y. | 8.0 | 0.18 | 13.0 | 0.18 | 17.0 | 0.19 |
| | Spores | 21.0 | 0.18 | 29.0 | 0.19 | 31.0 | 0.19 |

Perhaps this association between differential responses to egg-yolk treatment and resistance, as illustrated here between host strains, also holds true between host ages. If this is true then we must consider the young NIH rats to be more resistant to infection than the older NIH rats, because the effects of egg-yolk treatment of the spores are greater in the young NIH rats. These deductions agree with some of the septicemic data we have between ages, since among NIH rats the young die with a lower terminal concentration than the old. However, our data are too few and varied to answer this question adequately.

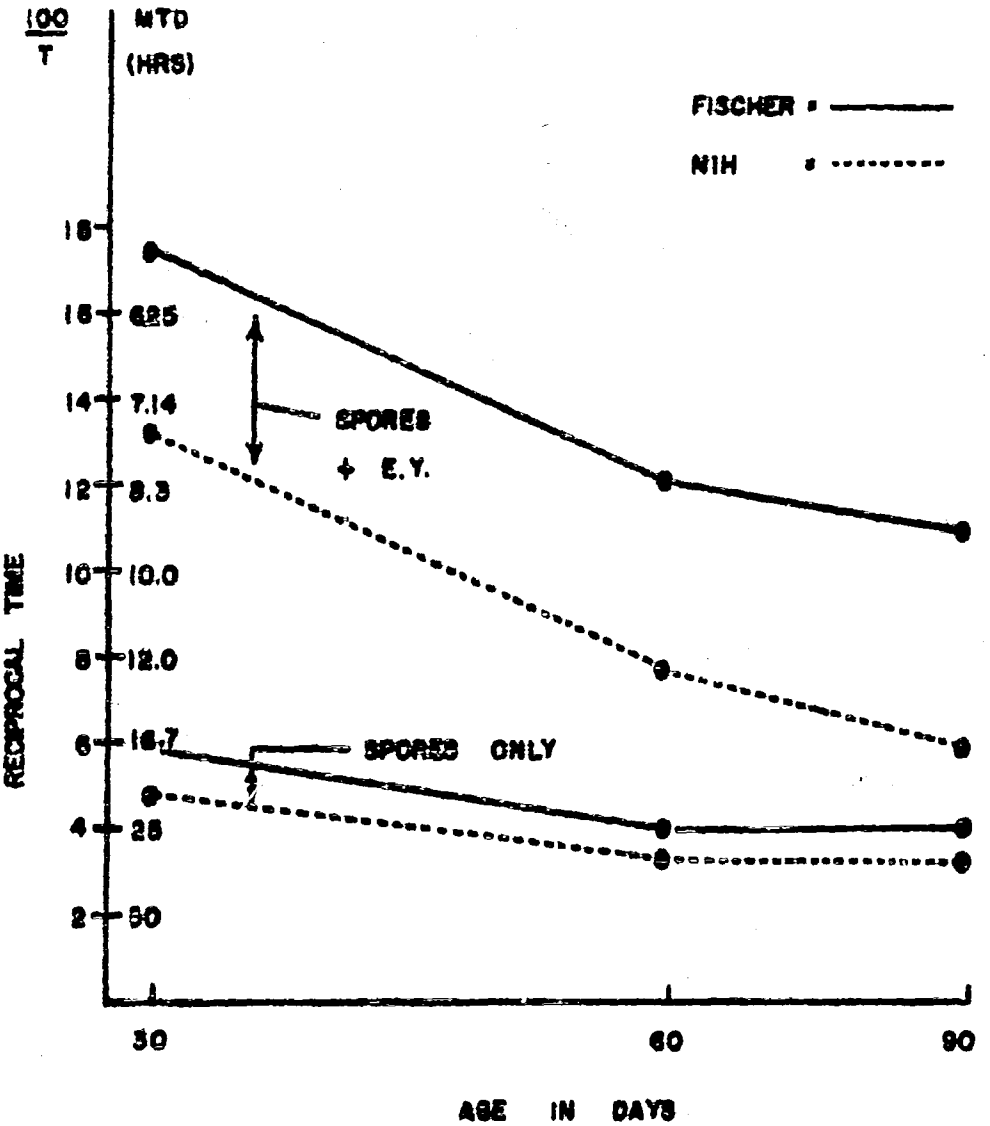


Figure 4. Time-To-Death of the Fischer and NIH Black Rats by Challenge Treatment and Age of Rat.

## IV. DISCUSSION

Three important concepts arise from these studies, a reaffirmation of Bennet and Beeson's[8] estimation of the value of a study of septicemia, the development of the idea that resistance to anthrax consists not only of resistance to the organism but also susceptibility to toxin, and the possibility that resistance to the bacilli and toxin need not be concurrent.

Egg-yolk enhancement of the anthrax spores was shown to change parameters that were relatively independent of toxin, but to leave unchanged those parameters of infection strongly influenced by toxin. This phenomenon was revealed in comparisons between control and treated animals and in comparisons between the two rodent species.

Conclusions about resistance to anthrax based on observations of response time alone are incomplete and may be misleading. That is, one species of animal that appears from its greater time-to-death to be more resistant to anthrax than other species may actually not be less resistant to infection by the organism but actually less susceptible to toxin. This is indicated by the greater terminal concentration of bacilli in the blood of the longer-lived animals than in those animals that succumb relatively soon. The results clearly indicate the complexity of resistance and its separability into distinct categories for even a single disease such as anthrax. This concept of both a bacterial and toxin resistance to anthrax can explain the fact that the effects of egg-yolk treatment of the spores on the mean time-to-death were greater on relatively resistant hosts (Fischer rats) than on relatively nonresistant hosts (NIH rats).

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